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Sequence analysis of *CXCR4* gene derived from feline lentivirus surviving cells

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Abstract

The feline 3201-S cell line was established from cells that survived productive infection with feline immunodeficiency virus (FIV). We have recently shown that 3201-S cells are free of FIV DNA, and are refractory to FIV reinfection. In addition, while the cells express CXCR4, a co-receptor for FIV infection, they are unresponsive to the CXCR4 ligand. The present study reports that 3201-S cells encode distinct mutations in the *CXCR4* gene. It would appear that 3201 cells are likely to be heterogeneous, consisting of phenotypically diverse mixed populations resulting from genetic mutation, suggesting that this defect can render the CXCR4 receptor expressed in 3201-S cells biologically dysfunctional.

Keywords : Feline immunodeficiency virus , 3201 , 3201-S , CXCR4 , Sequence analysis

Feline immunodeficiency virus (FIV) is a lentivirus associated with immunosuppressive disease in cats. It is similar to human immunodeficiency virus type 1 (HIV-1), although the viruses are genetically distinct [1,2]. Like HIV-1, FIV utilizes CXCR4, a member of the chemokine group of G-protein-coupled receptor family, as virus-entry co-receptor for infection. There is evidence of shared chemokine receptor use between HIV-1 and FIV [3]. CXCR4 is a seven transmembrane protein with an extracellular N-terminus, three extracellular loops (ECLs), three intracellular loops (ICLs) and an intracellular C-terminal tail [4]. Although the second extracellular loop (ECL2) has been reported to determine its function as a co-receptor for FIV [5], details of the mechanism of FIV entry in to cells remain to be elucidated,

In previous studies, we found that the feline thymic lymphoma 3201 cell line [6] was highly susceptible to the cytopathic effects of cell culture-adapted Petaluma strain of FIV [7]. However, after a productive infection associated with extensive cell killing, a few surviving cell populations remained. The surviving cells, designated as 3201-S, were shown to harbor no viral DNA as a proviral state [8]. We found that both 3201 and 3201-S cells express CXCR4, although the parental uninfected 3201 cells could be distinguished from 3201-S cells, as the 3201 cells seem to express much higher levels of CXCR4, as observed by fluorescence intensity. Of particular interest is the fact that 3201-S cells were unable to support the viral replicative cycle after reinfection with FIV. The presence of reverse-transcribed FIV DNA in the FIV-reinfected 3201-S cells early postinfection, albeit at an extremely low frequency, suggests that 3201-S cells are

insignificantly re-infectable by FIV as revealed by the real-time quantitative PCR analysis using extracted DNA from the cells, however, FIV failed to spread in 3201-S cells as demonstrated by the lack of any persistent presence of viral DNA. Furthermore, the stromal cell - derived factor - 1 α (SDF-1 α) stimulates calcium mobilization in 3201 but not in 3201-S cells, suggesting that the receptor is not functional in 3201-S cells [8]. These findings led us to hypothesize that phenotypic heterogeneity exists in the original 3201 cells and their heterogeneous expression of CXCR4 may influence CXCR4-dependent FIV entry and responsiveness to SDF-1 α . This brief report describes a study of sequence analysis of CXCR4 *gene* which we carried out to better understand the nature of CXCR4 dysfunction in 3201S cells, and their resistance to sustained FIV infection.

In order to determine the cause of the functional difference of CXCR4, the nucleotide sequences coding for CXCR4 in the two 3201-derived cell lines were examined. Total RNA was extracted from cultured 3201 and 3201-S cells using RealTime ready Cell Lysis Kit (Roche Diagnostics Japan, Tokyo). The RNA was reverse transcribed using Transcriptor Universal cDNA Master (Roche Diagnostics Japan, Tokyo) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using Pyrobest DNA polymerase (Takara Bio Inc, Otsu, Japan) and specific oligonucleotide primers for CXCR4; a sense primer ACCATGGACGGGTTTCGTATATAC (ATG corresponding to the start codon of the gene) and an antisense primer TTAGCTGGAGTGAAACTTGAAGA (TTA being complementary to the stop

codon of the gene), producing 1065 bases long cDNA. The PCR products were subjected to DNA sequence analysis. The DNA sequencing was performed using BigDye terminator cycle sequencing kit, and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo).

The nucleotide sequences coding for *CXCR4* in the two 3201-derived cell lines showed a 15 bp deletion in the *CXCR4* genes in both cell types [3], in comparison with a published sequence (GenBank accession # U67558). The deletion spanned amino acid residues 227 to 231 in a region corresponding to ICL3, which is associated with the domain required for Gi-dependent signaling [4] (Fig. 1). Since 3201 cells showed a flux of Ca^{2+} in response to SDF-1 α and susceptibility to FIV infection [8], the residues Leu-Ser-His-Ser-Lys do not appear to be essential for the biological function of the *CXCR4* protein.

More importantly, insertion of a thymidine base within the ECL3 region at the position between nucleotides 831 and 832 in the *CXCR4* gene of 3201-S cells has been noted. This insertion causes a nonsense frame shift leading to an early stop codon at AA position and thus truncation of the *CXCR4* receptor (Fig. 2). A predicted histidine residue (position 277) in 3201 cells was replaced by an alanine residue in 3201-S cells, resulting in a putatively truncated protein of 277 amino acids, whereas *CXCR4* from parental 3201 cells encoded a protein of 348 amino acids (Fig. 1). Although ECL2 is the principle determinant of co-receptor function, for

HIV-1 [9], and FIV [5], the adjacent loops such as ECL1 and ECL3 may help keep the ECL2 in the appropriate conformation for CXCR4 to support viral entry.

Our earlier observation that 3201-S cells can be re-infected by FIV, although inefficiently, but that the virus is unable to maintain the proviral state in these cells [8] is interesting in light of herein presented results. Our findings suggest that the truncated form of the CXCR4 protein (frame shift-induced deletion of the last 71 amino acids) may still exhibit conformational structures required for FIV entry, even though this mutated CXCR4 is very weakly expressed at the surface of the cells. In contrast, the truncated CXCR4 seems to have lost its function as chemokine receptor, since 3201-S cells did not respond to SDF-1 α . This finding is indicative for the coreceptor function of CXCR4 being independent of its function in chemokine signaling. This is in agreement with a previous report [10].

It remains unclear whether the mutated CXCR4 is the only reason for the FIV resistance in the 3201-S cells. It is necessary to study whether the exogenous expression of CXCR4 after transfecting the parental 3201 CXCR4 into the 3201-S cells can render the cells susceptible to FIV infection.

To our knowledge, this is the first report on non-synonymous mutations within the *CXCR4* gene of 3201-S cells. These mutations affect the third extracellular loop and negatively affect the chemokine receptor function of CXCR4. It appears that 3201 are genetically heterogeneous cells, as they are constituted of a mixed population of cells expressing

biologically functional and mutation-induced dysfunctional CXCR4 receptors.

These findings provide new insights into the natural history of FIV infection, and may be useful in the development of new prophylaxis strategies against FIV infection.

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Figure Legends

Figure 1. Comparison of the predicted amino acid sequences of feline *CXCR4* in 3201 and 3201-S cells. Numbers indicate the position of the amino acid. The character . denotes an identical amino acid, and - denotes the residue that has been deleted.

ICL3, intracellular loop 3 ; ECL3, extracellular loop 3.

Figure 2. CXCR4 mutation by polymerase chain reaction and direct DNA sequencing. The sequence difference is caused by a T addition in 3201-S cells, which generates a mutated amino acid sequence causing a STOP.

CXCR4-aa	1:MDGFRIYPSDNYTEDDLGSGDYDSMKEPCFREENAHFNRIFLPTVYSIIIFLTGIVGNGLV	60
3201	1:.....	60
3201-S	1:.....	60
CXCR4-aa	61:ILVMGYQKKLRSMTDKYRLHLSVADLLFVLTLPFWAVIDAVANWYFGKFLCKAVHVIYTVN	120
3201	61:.....	120
3201-S	61:.....	120
CXCR4-aa	121:LYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVYVGVWIPALLLTIPDFIFANVRE	180
3201	121:.....	180
3201-S	121:.....	180
		<u>ICL3</u>
CXCR4-aa	181:ADGRYICDRFYPSDSWLNVFQFQHIMVGLILPGIVILSCYCIISKLSHSKGYQKRKALK	240
3201	181:.....-----	235
3201-S	181:.....-----	235
		<u>ECL3</u>
CXCR4-aa	241:TTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFEFSTVHKWISITEALAFFHCCLNP	300
3201	236:.....	295
3201-S	236:.....A-----	277
CXCR4-aa	301:ILYAFLGAKFKTSAQHALLTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS	353
3201	296:.....	348
3201-S	:-----	277

Figure 1.

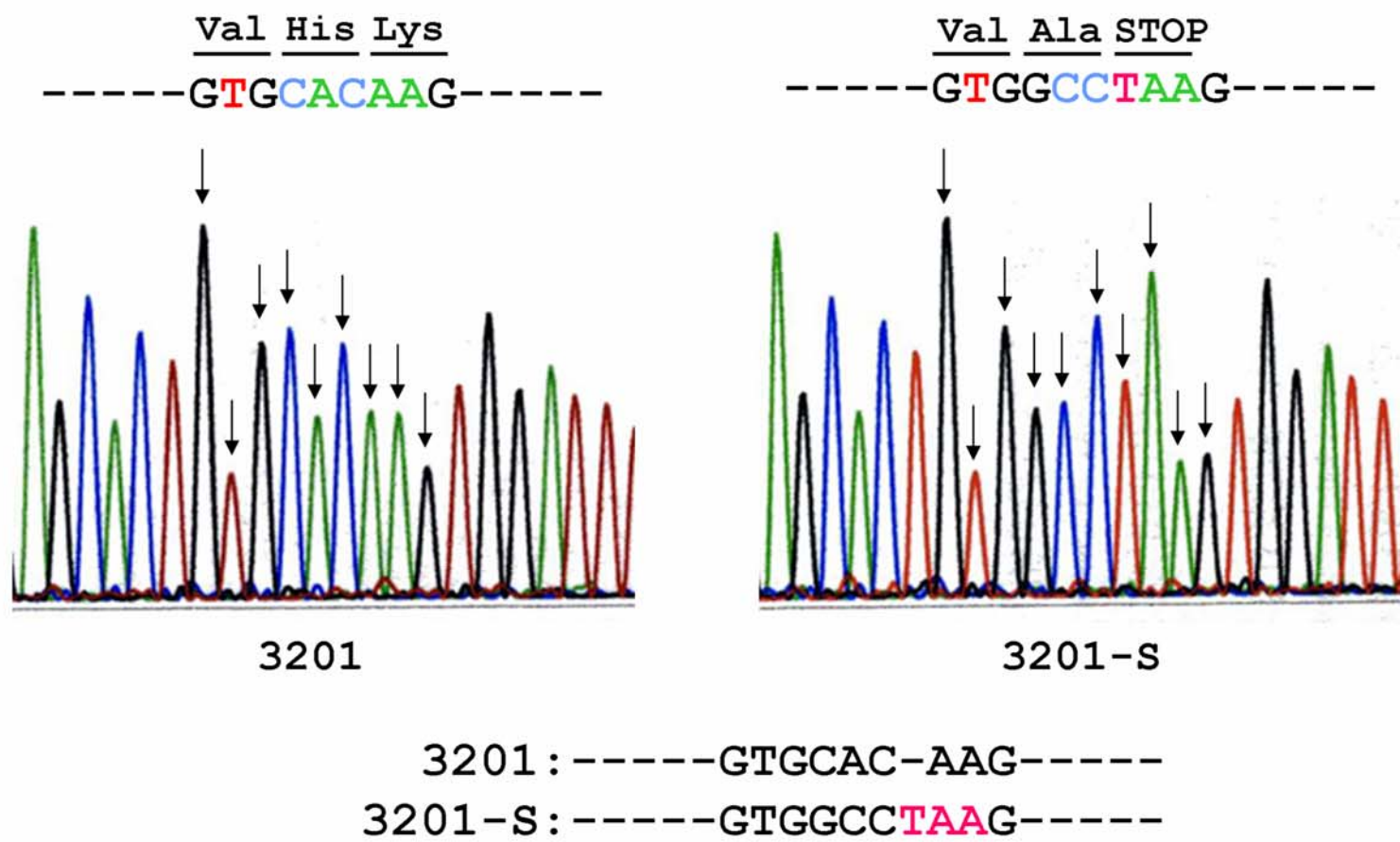


Figure 2.